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Pharmacokinetics of poly(hydroxyethyl-L-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration

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Abstract

'Stealth' liposomes with a poly(ethylene glycol) (PEG) coating are frequently studied for drug delivery and diagnostic purposes because of their prolonged blood circulation kinetics. However, several recent reports have demonstrated that PEG-liposomes are rapidly cleared at single low lipid doses (<1 μmol/kg) and upon repeated administration (time interval between the injections 5 days–4 weeks). Recently, poly(amino acid)-based stealth liposome coatings have been developed as alternative to the PEG-coating. In this study, the pharmacokinetic behavior of liposomes coated with the poly(amino acid) poly(hydroxyethyl-L-asparagine) (PHEA) was evaluated at low lipid doses and upon repeated administration in rats. Blood circulation times and hepatosplenic localization of PHEA-liposomes were assessed after intravenous injection. When administered at a dose of 0.25 μmol/kg or less, PHEA-liposomes showed significantly longer blood circulation times than PEG-liposomes. A second dose of PHEA-liposomes 1 week after the first injection was less rapidly cleared from the circulation than a second dose of PEG-liposomes. Although the mechanisms behind these observations are still not clear yet, the use of PHEA-liposomes appears beneficial when single low lipid doses and/or repeated dosing schedules are being applied.

Keywords: Long-circulating liposomes; Pharmacokinetics; Poly(ethylene glycol); Poly(amino acid)s; Accelerated blood clearance; Repeated administration

1. Introduction

Liposomes have been extensively studied for drug delivery and diagnostic purposes [1]. Their ability to extravasate at sites with increased vascular permeability, for example in solid tumors and inflamed tissue, enables selective targeting to these sites (enhanced permeability and retention (EPR) effect) [2]. Steric stabilization of liposomes with hydrophilic polymers such as poly(ethylene glycol) (PEG) results in a reduced uptake by cells of the mononuclear phagocyte system (MPS) leading to prolonged circulation times and thereby to an increased localization in the pathological site [3–5]. With several formulations on the market and in clinical trials, and numerous more in preclinical evaluation, PEG-liposomes remain the gold standard for long-circulating nanoparticles [5].

There are, however, several drawbacks in the application of PEG-liposomes, among which pharmacokinetic irregularities observed in animal models at low lipid doses and upon repeated administration [6–12]. In humans, irregularities at low lipid doses have been reported [7]. Although PEG-liposomes show dose-independent pharmacokinetics over a broad lipid dose range (4–400 μmol/kg) [13], this does not hold true at much lower lipid doses which are utilized for diagnostic imaging of e.g. tumors and sites of inflammation [7,14]. Several studies have shown that PEG-liposomes are rapidly cleared at such low lipid doses [6,7]. In rats, lipid doses lower than 0.03 μmol/kg were prone to accelerated clearance from the bloodstream. In humans, this phenomenon was observed at a lipid dose of 0.1 μmol/kg [7]. Despite several attempts to unravel the underlying cause, the mechanism of this phenomenon is still poorly understood. The faster disappearance from the circulation is accompanied by an increased accumulation in liver and spleen suggesting a role for hepatosplenic macrophages. Therefore, the

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involvement of a limited pool of opsonic proteins present in the circulation has been discussed, which induces rapid clearance of PEG-liposomes at low lipid doses and is depleted at higher liposome doses [14].

Besides at low lipid doses, rapid clearance was also observed upon repeated administration of PEG-liposomes [14]. This phenomenon has been referred to as 'accelerated blood clearance' (ABC). Several groups reported that a second dose of PEG-liposomes was rapidly cleared when administered within a certain time interval after the first dose; this was seen in different animal species [8–12]. In rats, dramatically decreased circulation times and elevated hepatosplenic uptake were found in case of ^{99m}Tc-labeled PEG-liposomes at a dose of 5 μmol/kg with an interval of 5 days to 4 weeks between the injections [9]. Time interval, doses, and liposome characteristics were different in the studies in which this ABC phenomenon was observed. Liposome size, surface charge, PEG density, and PEG molecular weight were reported to be of importance [14]. At doses higher than 50 μmol/kg pharmacokinetic irregularities did not occur in rats. Also in the case of repeated administration, the mechanism behind the observed pharmacokinetic changes has not been revealed yet. The involvement of serum factors was suggested, however, there is no consensus about their identity [14].

Recently, biodegradable poly(amino acid) (PAA)-lipid conjugates were successfully applied to prolong liposome circulation times. Circulation kinetics and biodistribution pattern of PAA-liposomes were similar to those of PEG-liposomes after single-dose administration of 25 μmol/kg [15,16]. The chemical nature of PAA, however, is very different from PEG. Therefore, it is likely that there are also differences in the nature of the steric stabilization layers formed by the respective materials on the surface of the liposomes. This, in turn, may lead to differences in pharmacokinetic behavior when administered repeatedly or at low lipid doses.

Both the rapid clearance at low lipid doses and upon repeated administration limit the applicability of PEG-liposomes. In the present study we investigated in rats the pharmacokinetic behavior of liposomes prepared with the poly(amino acid)-lipid conjugate poly(hydroxyethyl-L-asparagine)-succinyldioctadecylamine (PHEA-DODASuc) at low lipid doses and upon repeated administration. The results were compared to those obtained with PEG-liposomes. PHEA-liposomes appeared to show superior pharmacokinetic properties at low lipid doses and upon repeated administration.

2. Materials and methods

2.1. Materials

PHEA-DODASuc (Fig. 1, average MW = 3000 Da, determined by NMR and MALDI-ToF MS, corresponding with an average degree of polymerization of 15) was synthesized as described previously [15]. Dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG) and polyethylene glycol-distearoylphosphatidylethanolamine (PEG-DSPE) were purchased from Lipoid GmbH, Ludwigshafen, Germany. Cholesterol was obtained from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands. [^{3}H]-Cholesteryl oleylether was a product of Amersham, Roosendaal, The Netherlands. Ultima Gold liquid scintillation cocktail and Solvable tissue solubilizer were purchased from Perkin Elmer BioScience B.V., Groningen, The Netherlands. All other reagents were of analytical grade.

2.2. Animals

Male outbred Wistar rats, weighing approximately 250 g, were obtained from Harlan Nederland (Horst, The Netherlands). They were housed in groups of 4 and had free access to water and rat chow. All animal experiments were performed according to national regulations and approved by the local animal experiments ethical committee.

2.3. Liposome preparation

Liposomes were prepared by a film-extrusion method [17]. For the pharmacokinetic study involving single low lipid doses, EPC/EPG liposomes (1:1) with 5 mol% of PHEA-DODASuc or PEG-DSPE were prepared at two different lipid concentrations (20 and 0.2 μmol total lipid/mL, total volume of 2.5 mL). Lipids were dissolved in ethanol in 50 mL round-bottom flasks. To each formulation 20 μL of the non-degradable lipid phase marker [^{3}H]-cholesteryl oleylether were added (approximately 740 kBq). Lipid films were obtained by evaporation of the solvent under a stream of nitrogen. The lipid films were hydrated in 5 mL HBS (5 mM HEPES, 0.9% NaCl, pH 7.4) yielding total lipid concentrations of 20 and 0.2 μmol/mL. Liposomes were sized by sequential extrusion through two stacked polycarbonate filters (Poretics, Livermore, CA, USA, 400, 200, and 100 nm pore size) with a high-pressure extrusion device. The formulation prepared at a concentration of 0.2 μmol/mL was divided into two equal parts, and one part was further diluted 1:10 in HBS, yielding a lipid concentration of 0.02 μmol/mL.

For the pharmacokinetic study involving repeated administration, liposomes composed of DPPC/cholesterol/coating polymer in a molar ratio of

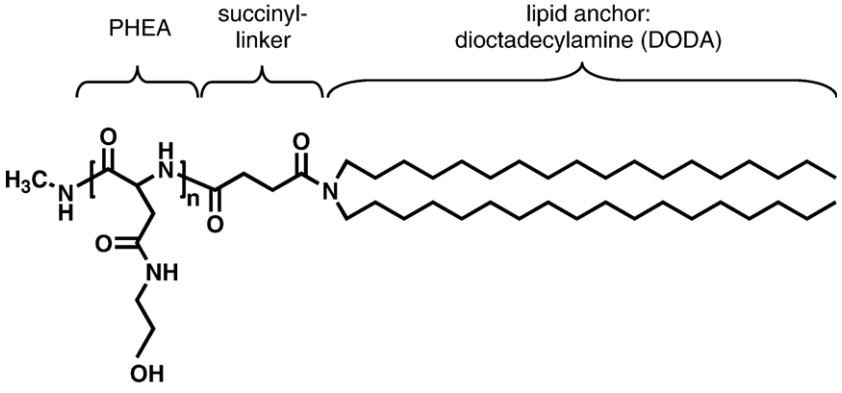

Fig. 1. Molecular structure of poly(hydroxyethyl-L-asparagine)-succinyldioctadecylamine (PHEA-DODASuc, $n \approx 15$).

1.85:1:0.15 were used. Liposomes were prepared as described above with lipid concentrations of 5 μmol/mL (for a lipid dose of 5 μmol/kg rat body weight) or 12.5 μmol/mL (for a lipid dose of 10 and 20 μmol/kg rat body weight). The [^{3}H]-cholesteryl oleylether label was added only to the liposome formulations to be monitored for circulation times and biodistribution. For the composition of the different formulations see Table 1.

2.4. Liposome characterization

The mean particle size and polydispersity index of the liposome dispersions (diluted 1:100 with HBS) were determined by dynamic light scattering using a Malvern ALV/CGS-3 Goniometer. The lipid content of the liposomal dispersions was assessed by phospholipid quantification according to Rouser et al. [18]. Radioactivity of the liposomal dispersions was assayed in an Ultima Gold liquid scintillation cocktail (dilution of 1:1000) and counted in a Packard Tricarb 2200 CA liquid scintillation counter. Liposome preparations were stored at 4 °C and used within 1 week after preparation.

2.5. Pharmacokinetics

For the pharmacokinetic study involving low lipid doses, single doses of the liposomal preparations containing 25, 0.25 or 0.025 μmol total lipid/kg body weight were administered via the tail vein. Blood samples of 150 μL were collected from the tail vein of each rat at the following time points: immediately after injection (opposite vein to injection vein) and at 1, 4, 8, 24, and 48 h after injection. For the pharmacokinetic study involving repeated administration, either no priming dose or a priming dose of 5, 10, or 20 μmol/kg body weight of non-labeled liposomes was given. Seven days later the corresponding labeled formulations were injected at the same dose. Blood samples of 150 μL were collected as described above immediately after injection (from the opposite tail vein) and at 1, 4, 8, 24, and 48 h after injection. To 100 μL of the blood samples, 100 μL Solvable tissue solubilizer and 100–200 μL 35% hydrogen peroxide to bleach the samples were added and samples were incubated at room temperature overnight. Samples were diluted in 10 mL Ultima Gold scintillation cocktail and counted for radioactivity with a Packard Tricarb 2200CA liquid scintillation counter.

For the study involving repeated administration, liver and spleen were dissected at 48 h post-injection and homogenized in 25 mL and 5 mL water, respectively, using an Ultra-Turrax T8 (IKA Labortechnik GmbH, Staufen, Germany) at 25000 rpm for approximately 30 s. To 0.5 mL of the liver homogenate and 1 mL of the spleen homogenate, 200 μL of Solvable tissue solubilizer and 200 μL 35% hydrogen peroxide were added and incubated at room temperature until the tissue was dissolved (usually overnight). After adding 10 mL of Ultima Gold scintillation cocktail, radioactivity was counted as described for the blood samples. Besides tissue and blood samples, also the radioactivity of the injected amount of liposomes was counted.

2.6. Data analysis

Blood concentrations of liposomes at the different time points were calculated from the radioactivity of the blood samples as the percentage of the radioactivity measured immediately after injection (% injected dose). For the blood samples, a Q-test for identification of outliers was applied. From the blood concentration–time curves, area under the curve values were calculated for the analyzed time interval from zero to 48 h (AUC_{0-48h} [μmol/kg × h]). Differences in AUCs were compared by a two-tailed Student's *t*-test assuming equal variances with a 95% confidence interval. Differences were considered significant when the *p*-value was less than 0.05. The percentage of the injected dose in the organs was calculated by dividing the total radioactivity of the organs by the radioactivity of the injected liposome amount.

3. Results

3.1. Liposome characteristics

Table 1 shows the characteristics of the liposome formulations used in this study. The mean size of the liposomes ranged from 110 to 150 nm. The polydispersity index (PD) was ≤ 0.12, indicating a narrow size distribution. All preparations were physically stable during storage at 4 °C for at least 1 week.

3.2. Pharmacokinetics at low lipid doses

To evaluate the possibility of alterations in circulation time of PHEA-liposomes occurring when low lipid doses are applied, radiolabeled PHEA- (EPC/EPG/PHEA-DODASuc) and PEG-liposomes (EPC/EPG/PEG-DSPE) were injected i.v. at lipid doses of 25, 0.25, and 0.025 μmol/kg. The resulting circulation kinetics and the AUC_{0-48h} values are presented in Fig. 2. For the high dose of 25 μmol/kg, the circulation behavior of the PEG- and the PHEA-liposomes were comparable, as reflected by similar blood concentration–time curves and AUC_{0-48h} values. At the lower dose of 0.25 μmol/kg, PEG-liposomes were cleared more rapidly from the bloodstream than at 25 μmol/kg. PHEA-liposomes, however, show similar circulation kinetics after injection of 25 and 0.25 μmol/kg. Twenty-four hours after administration of 0.25 μmol/kg, 24.5% of the PHEA-liposomes were detected in the blood circulation, versus

Table 1
Liposome characteristics

Application	Formulation	Composition (molar ratio)	Mean size [nm]	PD
Lipid dose study				
Lipid dose: 25 μmol/kg	PEG-liposomes	EPC/EPG/PEG-DSPE 1.425:1.425:0.15	140	0.07
	PHEA-liposomes	EPC/EPG/PHEA-DODASuc 1.425:1.425:0.15	125	0.10
Lipid dose: 0.25 and 0.025 μmol/kg	PEG-liposomes	EPC/EPG/PEG-DSPE 1.425:1.425:0.15	130	0.10
	PHEA-liposomes	EPC/EPG/PHEA-DODASuc 1.425:1.425:0.15	110	0.12
Repeated administration study				
Non-labeled dose (priming): 5 μmol/kg	PEG-liposomes	DPPC/cholesterol/PEG-DSPE 1.85:1:0.15	125	0.04
	PHEA-liposomes	DPPC/cholesterol/PHEA-DODASuc 1.85:1:0.15	125	0.06
Non-labeled dose (priming): 10 and 20 μmol/kg	PEG-liposomes	DPPC/cholesterol/PEG-DSPE 1.85:1:0.15	135	0.06
	PHEA-liposomes	DPPC/cholesterol/PHEA-DODASuc 1.85:1:0.15	130	0.04
Labeled dose: 5 μmol/kg	PEG-liposomes	DPPC/cholesterol/PEG-DSPE 1.85:1:0.15	145	0.06
	PHEA-liposomes	DPPC/cholesterol/PHEA-DODASuc 1.85:1:0.15	145	0.02
Labeled dose: 10 and 20 μmol/kg	PEG-liposomes	DPPC/cholesterol/PEG-DSPE 1.85:1:0.15	140	0.05
	PHEA-liposomes	DPPC/cholesterol/PHEA-DODASuc 1.85:1:0.15	135	0.06

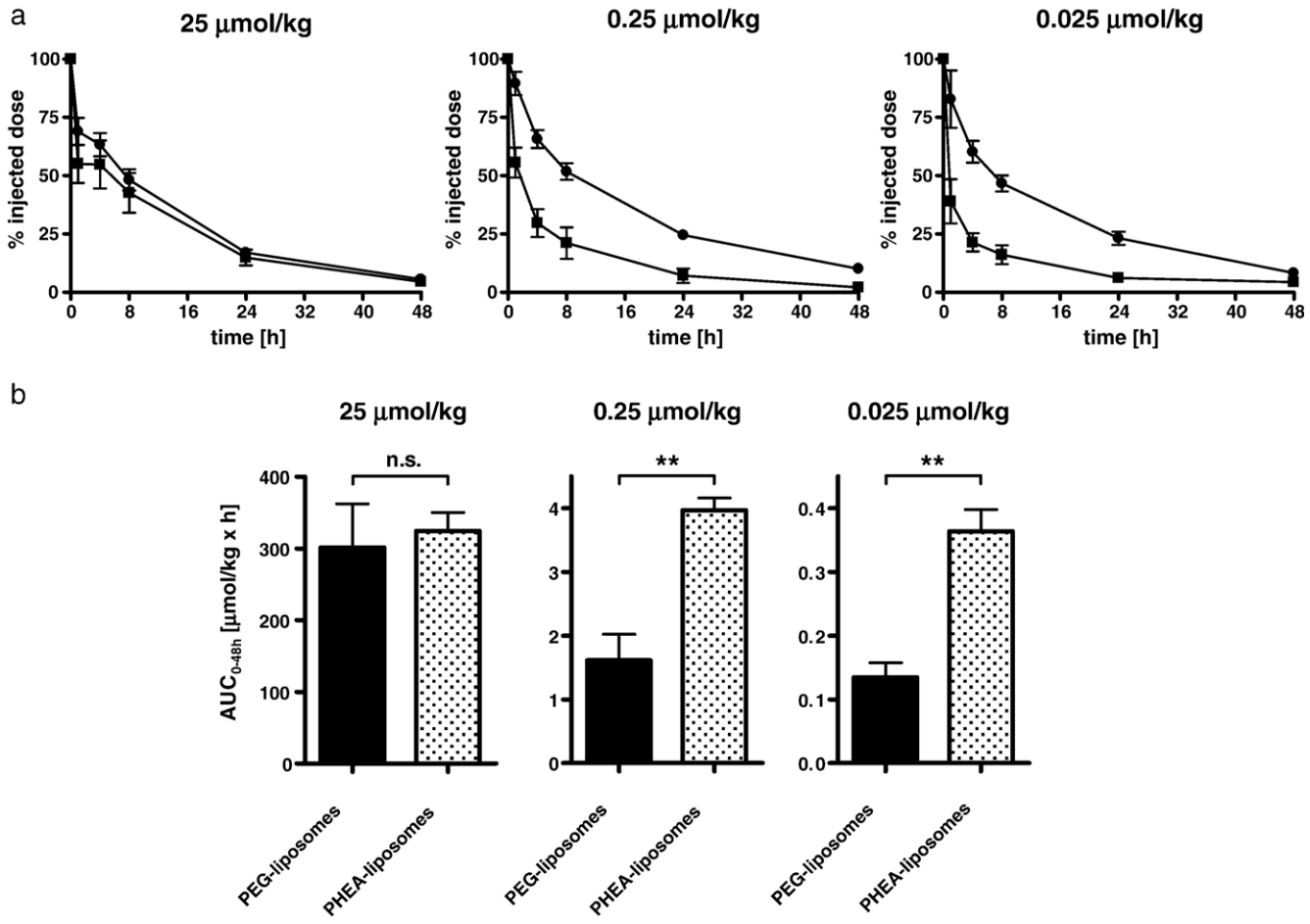

Fig. 2. Comparative pharmacokinetics of PHEA- and PEG-liposomes at lipid doses of 25 μmol/kg, 0.25 μmol/kg, and 0.025 μmol/kg. (a) Circulation kinetics of PHEA- (EPC/EPG/PHEA-DODASuc) (●) and PEG-liposomes (EPC/EPG/PEG-DSPE) (■) (% injected dose vs. time). (b) Area under the curve ($AUC_{0–48h}$) values calculated from (a). All results are expressed as mean±SD (n=3–4). **p<0.01; n.s.=not significant.

only 7.1% of the PEG-liposomes. The $AUC_{0–48h}$ value of the PHEA-liposomes was approximately twofold higher than that of the PEG-liposomes. At the lowest dose of 0.025 μmol/kg, the difference in pharmacokinetics of PHEA- and PEG-liposomes was maintained; the difference in the $AUC_{0–48h}$ values was about 2.5-fold.

3.3. Pharmacokinetics and biodistribution at single and repeated administration

The effect of repeated administration of PHEA-liposomes (DPPC/cholesterol/PHEA-DODASuc) on pharmacokinetics and biodistribution was evaluated by administering liposomes i.v. either as a single dose (labeled dose) or injecting twice (priming and labeled dose) with a time interval of 7 days between the two injections. Lipid doses of 5, 10, and 20 μmol/kg were investigated. For comparison, PEG-liposomes (DPPC/cholesterol/PEG-DSPE) were also studied using the same experimental conditions. Fig. 3 presents the blood concentration–time curves and the ratios of the $AUC_{0–48h}$ of the second injection to the $AUC_{0–48h}$ of the first injection ($AUC_{2nd/1st}$). If the $AUC_{2nd/1st}$ equals one, no differences in circulation times between the liposomes given at the second and the first injection were observed. If the $AUC_{2nd/1st}$ is smaller than one, the liposomes given at the second injection were cleared more rapidly from the circulation than those given at the first injection. At the lipid dose of 5 μmol/kg the first dose of PEG-liposomes yielded prolonged circulation times, whereas liposomes administered at the second dose were cleared very rapidly, with only 2.3% of the liposomes left in the circulation at 1 h after administration. The $AUC_{2nd/1st}$ was 0.04, indicating that the $AUC_{0–48h}$ of the second injection was very small as compared to the $AUC_{0–48h}$ of the first injection. In the case of administration of 5 μmol/kg PHEA-liposomes, the second dose circulated significantly longer than the second dose of PEG-liposomes, with 52.8% of the liposomes still circulating in the blood 1 h after injection. The $AUC_{0–48h}$ of the second injection was about half of the $AUC_{0–48h}$ of the first injection ($AUC_{2nd/1st}$=0.47). While at 10 μmol/kg the second dose of PEG-liposomes was still very rapidly removed from the circulation, the second dose of PHEA-liposomes showed enhanced circulation times when compared with the lower dose of 5 μmol/kg. The $AUC_{2nd/1st}$ of the PHEA-liposomes was 0.67. In case of the dose of 20 μmol/kg, the second dose of PHEA-liposomes circulated as long as the first dose, as reflected by an $AUC_{2nd/1st}$ of one. The second dose of PEG-liposomes

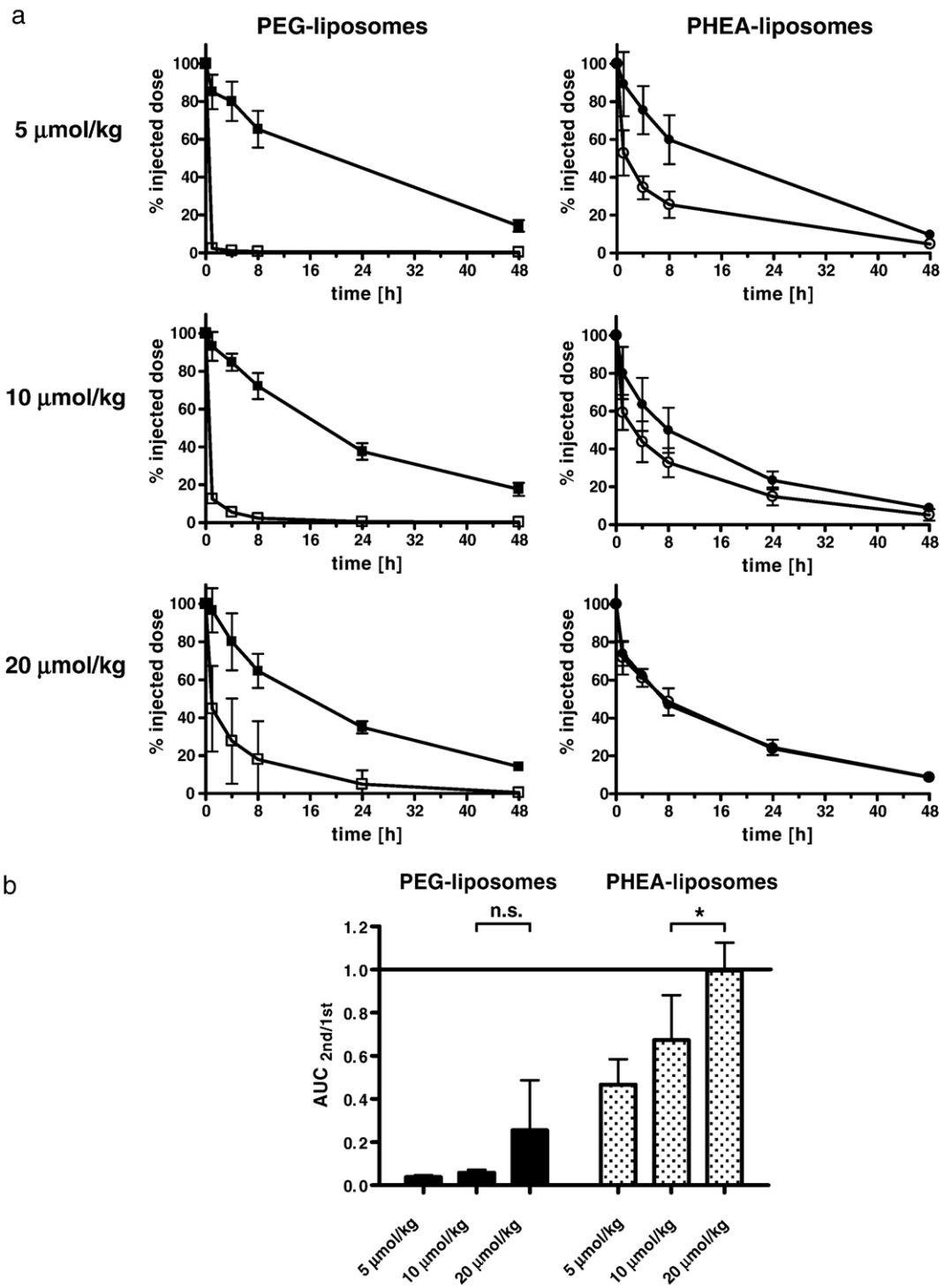

Fig. 3. Effect of the lipid dose on pharmacokinetic behavior of PHEA- and PEG-liposomes after first and second injection. (a) Circulation kinetics of PHEA- (DPPC/cholesterol/PHEA-DODASuc) and PEG-liposomes (DPPC/cholesterol/PEG-DSPE) (% injected dose vs. time). Closed symbols represent the results after the first injection, open symbols those after the second injection of liposomes. (b) Ratio of the AUC_{0-48h} of the second injection to the AUC_{0-48h} of the first injection ($AUC_{2nd/1st}$) at the different lipid doses. AUC_{0-48h} values were calculated from (a). Filled bars represent the $AUC_{2nd/1st}$ of PEG-liposomes, dotted bars represent the $AUC_{2nd/1st}$ of PHEA-liposomes. All results are expressed as mean±SD (n=3–4). *p<0.05; n.s.=not significant.

circulated longer at this dose level ($AUC_{2nd/1st}$=0.25) as compared to the second dose of 5 and 10 µmol/kg, but by far shorter than the second dose of PHEA-liposomes.

Fig. 4 shows the localization of PEG- (DPPC/cholesterol/PEG-DSPE) and PHEA-liposomes (DPPC/cholesterol/PHEA-DODASuc) in liver and spleen at 48 h after administration. The ratios of the percentage of injected dose of the second injection to the percentage of the injected dose of the first injection recovered in the two organs were calculated (% $ID_{2nd/1st}$). If this ratio is smaller than one, the second dose was taken up by the

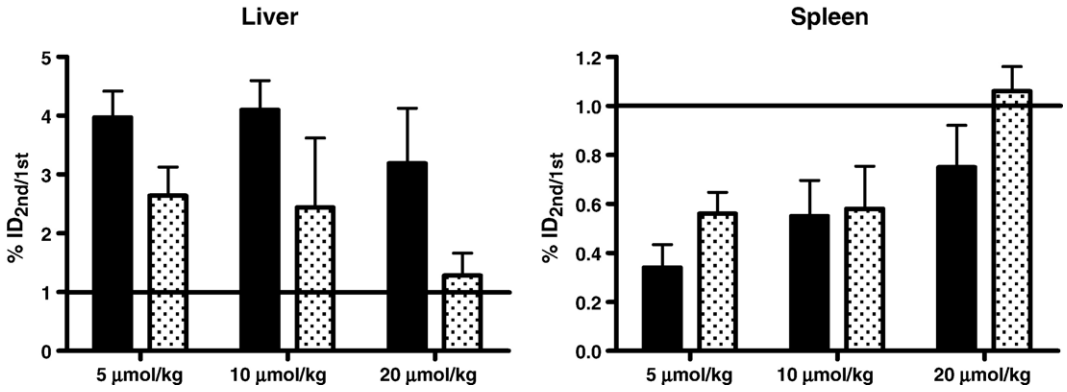

Fig. 4. Hepatosplenic localization of PHEA- and PEG-liposomes at 48 h after injection: ratio of the percentage of injected dose of the second injection to the percentage of injected dose of the first injection (% $ID_{2nd/1st}$). Filled bars represent the % $ID_{2nd/1st}$ of PEG-liposomes (DPPC/cholesterol/PEG-DSPE), dotted bars represent the % $ID_{2nd/1st}$ of PHEA-liposomes (DPPC/cholesterol/PHEA-DODASuc). All results are expressed as mean ± SD (n = 3–4).

organ to a lesser extent than the first dose. If it is larger than one, a larger fraction of the second dose was taken up as compared to the fraction of the first dose. Remarkably, the % $ID_{2nd/1st}$ values observed for the liver are above one for both formulations at all dose levels, indicating that the second dose of liposomes is primarily taken up by the liver, whereas the spleen is less involved in processing of the second dose when compared to the first dose as demonstrated by splenic % $ID_{2nd/1st}$ values below one. The differences in biodistribution between the first and the second dose diminished at higher doses, especially in the case of the PHEA-liposomes. In both organs the % $ID_{2nd/1st}$ of 20 µmol/kg PHEA-liposomes is close to one, showing that there are no differences in liver and spleen distribution between the first and the second injection at this dose level.

4. Discussion

In recent years it has been recognized that PEG-liposomes are not as 'stealth' in the biological environment as was generally assumed. Although stealth coatings reduce rapid clearance of the liposomes from the circulation, extensive binding of blood proteins to the liposome surface does occur after intravenous administration [19]. Hence, the long circulation time of PEG-liposomes is not necessarily a direct result of a non-specific reduction of protein adsorption as often claimed, but likely related to a combination of several mechanisms [19,20]. For example, so-called dysoponins might contribute to the stealth behavior. They have been hypothesized to bind to the liposome surface in the circulation, thereby repelling binding of opsonic factors, and as a consequence reducing the uptake of the liposomes by phagocytic cells. Besides the observation of extensive binding of blood proteins to the PEG-liposome surface, pharmacokinetic irregularities, such as the ABC phenomenon, in case of repeated administration of PEG-liposomes and enhanced clearance at low lipid doses additionally demonstrate that PEG-liposomes are not 'invisible' in vivo [14].

The present study was conducted to investigate if liposomes coated with the new poly(amino acid)-coating PHEA lack the pharmacokinetic irregularities observed for PEG-liposomes upon repeated administration and at low lipid dose. Our results with lipid doses ≤ 0.25 µmol/kg body weight clearly show that PHEA-liposomes have superior pharmacokinetic behavior at single low lipid doses as compared to PEG-liposomes. The rapid clearance of PEG-liposomes at low lipid doses is likely mediated by a pool of opsonic factors that interact with PEG-liposomes and induce phagocytosis [19]. At higher lipid doses, the influence of this relatively low amount of opsonic factors becomes more limited and the majority of the administered liposomes will be long-circulating. The higher the lipid dose, the less this opsonization effect will be detectable in the overall circulation profile. The theory of a limited pool of opsonins is in agreement with the generally observed finding that a small fraction of PEG-liposomes administered at higher lipid doses is rapidly cleared within 60 min after administration [14]. Our observation that low doses of PHEA-coated liposomes show relatively long circulation times suggests that the process of opsonization is rather different for PHEA- and PEG-liposomes. A lower affinity of the opsonins involved for the PHEA-liposomes as compared to PEG-liposomes could result in extended circulation times. Moreover, due to the very different chemical nature of the PHEA-molecule in comparison to PEG, the type and/or adsorbed amount of opsonins may be substantially different as a reflection of differences in chemical structure, hydrophilicity, length, and flexibility. Further investigations on the protein adsorption pattern will shed more light on the mechanism behind the superior circulation time of PHEA-liposomes at low lipid doses.

As aforementioned, repeated administration of PEG-liposomes can lead to rapid clearance of the subsequent injections as compared to the first injection (ABC phenomenon). Besides being favorable at single low lipid dose, PHEA-liposomes are also much less susceptible for the ABC phenomenon when compared to PEG-liposomes by showing superior circulation kinetics upon repeated administration at the dose range of 5–20 µmol/kg. A time interval of 7 days between the first and second injection was chosen in this study as at this interval the clearance of a second dose of PEG-liposomes was most rapid in rats [9]. At much longer or shorter time intervals the effect was attenuated. The ABC phenomenon can be induced by a variety of liposome formulations, including liposome types, which lack

a PEG-coating [8,21]. And the presence of a PEG-coating appeared also not to be an essential requirement for the rapid clearance of liposomes given as the second dose [8]. At present, there is no consensus about the mechanism underlying the ABC phenomenon. A role for hepatosplenic macrophages in the ABC phenomenon has been proposed [8,9]. Several studies have shown that in particular Kupffer cells of the liver take part in the clearance of a second dose of liposomes. Our data on the hepatosplenic distribution of the liposomes investigated are in line with these observations; uptake of the second dose by the liver was highly increased when the ABC phenomenon was induced. Transfusable humoral serum factors have been suggested to play a role, and also IgG or IgM have been proposed to be involved [9,22,23]. Recently, Ishida et al. reported on an IgM molecule with anti-PEG activity, which binds to a second dose of liposomes and subsequently activates the complement system [23]. Yet, as also non-PEGylated liposomes can induce the phenomenon, it is obvious that the mechanism behind the ABC phenomenon is more complex, and that PHEA- and PEG-liposomes behave differently in this regard.

The implications of the present findings for the use of the currently approved liposomal drug formulations (e.g. the doxorubicin containing PEG-liposome product Doxil®/Caelyx®) is not clear. However, the data do suggest that in the clinical setting, where repeated administration of liposomal drugs will often be required, the therapeutic efficacy might be compromised by an accelerated MPS clearance of repeatedly injected liposome-based therapeutics. Furthermore, a rapid and extensive uptake by hepatosplenic organs as observed after administration of PEG-liposomes may increase the toxicity towards these organs conferred by increased accumulation of the incorporated drugs.

In conclusion, although the mechanistic details are still largely obscure, this paper highlights that the use of PHEA-coated long-circulating liposomes may be beneficial when single low lipid doses and/or repeated dosing schedules are being applied.

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